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(71) Applicant: EDGE BIOSYSTEMS, INC. [US/US]; 19212 Orbit Drive, Gaithersburg, MD 20879 (US).

(72) Inventors: SEED, Brian; Edge Biosystems, Inc., 19212 Orbit Drive, Gaithersburg, MD 20879 (US). SHAO, Wen; Edge Biosystems, Inc., 19212 Orbit Drive, Gaithersburg, MD 20879 (US). SEED, John; Edge Biosystems, Inc., 19212 Orbit Drive, Gaithersburg, MD 20879 (US).

(74) Agents: KAGAN, Sarah, A. et al.; Banner & Witcoff, Ltd., 11th floor, 1001 G Street, N.W., Washington, DC 20001-4597 (US).

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(54) Title: MATRIX FOR RAPID PURIFICATION OF NUCLEIC ACIDS

(57) Abstract

A matrix suitable for single-step purification of nucleic acids from a cell lysate binds some contaminants and retards others. The nucleic acids pass through in the eluate.

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MATRIX FOR RAPID PURIFICATION OF NUCLEIC ACIDS

TECHNICAL FIELD OF THE INVENTION

This invention is related to the area of purification of nucleic acids.

BACKGROUND OF THE INVENTION

Numerous methods have been described for the purification of nucleic acids. These typically include phenol extraction, ion exchange chromatography, adsorption of DNA to glass powder, silica, and diatomaceous earth, PEG or ethanol precipitation and gel filtration (Reviewed by Kirby, Progr. Nucl. Acid Res. Mol. Biol. Vol. 3, p.1, 1964; Sambrook, Fritsch and Maniatis, Molecular Cloning, Vol, 1, Cold Spring Harbor Laboratory, Cold Spring Harbor, 1989; Ausubel, et. al., Current Protocols in Molecular Biology, Vol 1, John Wiley & Sons, New York, 1995). McCormack, U.S. 4,923,978 teaches the use of a solid phase material with a large surface area and a high concentration of mildly acidic hydroxyls, e.g. silica, for the removal of proteins from nucleic acids. Seed & Seed, EPO 0524800 teach the extraction of proteins from nucleic acids with aromatic polymers . While the purification of nucleic acids from enzymes and other reaction components used to modify DNA is relatively straightforward, DNA purification from crude biological samples is considerably more complex. The latter material contains large quantities of protein, lipid, carbohydrates and glycosylated macromolecules in addition to low molecular weight contaminants and in some cases detergents. Ion exchange techniques efficiently separate many common contaminants of these samples from DNA and may also separate different forms of DNA such as supercoiled plasmid from linear DNA *e.g.*, genomic DNA. The principle disadvantage of this technique is that the procedure is slow, typically involving gravity flow processing, and generates large volumes of fluid from which DNA

must be concentrated. The adsorption techniques (glass, silica, and diatomaceous earth), do not provide the same levels of purity, but are significantly faster than most ion exchange techniques and deliver the DNA in a small volume of low ionic strength buffer. Resin contamination of the sample and limitations in either binding capacity or recovery of DNA from the resin are typical disadvantages to the adsorption methods. These techniques also require significant inputs of time and effort due to the requirement of binding the DNA to the matrix, washing out contaminants, and elution. The composition of the wash buffer also can significantly impact the purity of the sample by contaminating the preparation with solvents and/or salts. The technique of extracting contaminants from the DNA requires fewer manipulations than the techniques described above but typically leaves many low molecular weight residues in the sample and consequently requires an alcohol precipitation step. The latter is undesirable for safety reasons, substantially increases processing time, and carries a risk of catastrophic sample loss. Kung, et al., EPO 0310251 teach the use of proteolytic enzymes in combination with gel filtration to purify DNA. This technique is not widely used and its effectiveness in removing all contaminants is not known. Because it requires the use of enzymes, the technique is time consuming and expensive, and also requires sample concentration steps such as alcohol precipitation. With increasing use of DNA in the diagnostic and sequencing industry and increased efforts to reduce exposure to organic solvents, there is an increased need for techniques for DNA sample preparation which are simple, fast, and yield high purity DNA and require no organic solvents.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a matrix for purifying nucleic acids from biological samples and delivering said nucleic acid in a buffer of known composition.

It is another object of the present invention to provide a method of purifying nucleic acids from biological samples and delivering the nucleic acid in a buffer of known composition using the matrix.

These and other objects of the invention are achieved by providing a matrix for purifying a desired nucleic acid having a defined minimum molecular weight. The matrix has the ability to bind to proteins and in some cases detergents but not nucleic acids. The matrix also retards the migration through the matrix of components having a an effective hydrodynamic radius smaller than that of nucleic acids of a minimum molecular weight. The matrix also is hydrated with water or a buffer of known composition.

In another embodiment of the invention a method is provided for purifying a desired nucleic acid having a defined minimum molecular weight. The method comprises the step of applying a preparation comprising the desired nucleic acid to a column containing a matrix having the ability to bind to proteins and in some cases to detergents but not to nucleic acids. The matrix retards the migration through the matrix of components having an effective hydrodynamic radius smaller than that of nucleic acids of a minimum molecular weight. The matrix is hydrated with water or a buffer of known composition in which the purified nucleic acid is delivered. These and other embodiments of the invention which will be apparent to those of skill in the art, provide improved methods for isolating nucleic acids from cells or other complex mixtures in a single step.

DETAILED DESCRIPTION

The inventors have discovered that by formulating a matrix which has properties associated with a number of distinct chromatography matrices, that a single step chromatographic separation can be used to provide very clean preparations of nucleic acids from biological and experimental samples. Using such a separation, one can obtain preparations which are suitable for multiple analytic and therapeutic uses. In addition, the nucleic acid can be eluted from the matrix in a minimum volume in water or a buffer of desired composition, thus obviating the need to precipitate for purposes of concentration or buffer

exchange in most circustances. The matrix of the present invention is typically provided in the form of a column from which the purified sample can be collected.

The matrix of the present invention has several important characteristics: it binds proteins; it may bind detergents; it retards the migration of molecules having a smaller hydrodynamic radius than the DNA molecule which is being purified; it does not bind to nucleic acids; it does not retard the migration of molecules having a hydrodynamic radius greater than of equal to the DNA molecule which is being purified; and it is hydrated with water or a buffer of known composition. All of these characteristics can be provided by a single physical component, or they may be provided by two or more components which are mixed together, or which are layered in a column.

Binding of proteins to the matrix can occur by any intermolecular interaction, such as affinity binding, ionic interactions, hydrophobic interactions, hydrogen binding, and Van der Waals interactions. In addition, it is desirable for some applications that the matrix bind to other substances which typically contaminate nucleic acid preparations, detergents in particular. These contaminants are frequently found in cell lysates produced by the alkaline detergent method (Birnboim and Doly, Nucl. Acids Res. 7, p.1513, 1979) and the contaminants may form aggregates which are difficult to separate from nucleic acids on the basis of size. A preferred material for protein binding, according to the invention is poly(4-hydroxystyrene), although any material known in the art to bind protein with high affinity for protein can be used. This protein binding material may not carry a surface positive charge which is accessible for binding by nucleic acids. By way of example, but without limitation, other suitable protein binding materials include silica, glass beads, diatomaceous earth, various metal and semiconductor oxides, beads or similar packing material prepared from protein-binding polymers such as polyvinylidenefluoride, nitrocellulose and polymers bearing uncharged or negatively charged protein binding dyes, and resins with affinity for particular proteins or which are substituted with molecules, e.g. antibodies, which bind

certain proteins with high affinity. Positively charged protein binding matrices can be used if the positive charges are sequestered within the matrix so that proteins having an effective hydrodynamic radius less than that of the minimum molecular weight nucleic acid are admitted, but all species having a larger radius are excluded.

Retardation of lower molecular weight substances is accomplished by using a "molecular sieve", *i.e.*, a substance which has pores of a defined size range such that only molecules smaller than a certain hydrodynamic radius will enter the pores and be retarded by such entry, whereas molecules with a hydrodynamic radius larger than the pore size will not enter and therefore their migration will not be retarded by the porous substance. The pore size which is useful for isolating DNA from crude biological samples is the same as is found in porous chromatographic media useful for fractionating globular proteins of 1×10^3 to 8×10^6. The matrix should be resistant to deformation and have a particle size in the range of 2.5 - 1000 microns, with a preferred particle size of 30-120 microns. The sieving matrices of the invention are cross-linked hydrophilic polymers or copolymers. These include by way of example but without limitation, dextran, agarose, surface modified silica, porous glass and substituted acrylamides. The precise degree of cross linking required to achieve the retention characteristics described above are well known to those of skill in the art. Preferred materials to use as a molecular sieve, *i.e.*, as the retarding component of the matrix are cross-linked dextrans or cross-linked acrylamides. Suitable commercially available products include Sephacryl ™ S-400HR (Pharmacia), Sephadex ™ G100 (Pharmacia) and BioGel P100 (BioRad). According to one preferred embodiment of the invention a singel component matrix for purification of nucleic acids comprises beads of a cross-linked hydroxylated aromatic polymer having a suitable pore size as defined above. According to another embodiment, a variety of components, each having a desirable characteristic as identified above, may be mixed and added to a column or layered onto a column. See Examples below. It is also possible to have separate components which may not have useful

sieving properties, and which do not bind DNA, but are useful for binding detergents and lipids, such as polystyrene.

Using the chromatographic matrix of the present invention one can rapidly purify nucleic acids from crude biological or experimental samples. Crude biological samples comprise cells or cell lysates or combinations thereof. Such combinations include by way of example but without limitation, blood, tissues, soil samples, body fluids, scrapings and plant materials. For example, cells can be lysed by physical, chemical or enzymatic means, or combinations thereof. When isolating DNA, RNase may be added to the lysis solution to facilitate the degradation of RNA and its subsequent separation from DNA on the basis of size. Lysis in the presence of RNase occurs most favorably with a salt concentration of 0.1 M or less and optimally with a salt concentration of 0.01 M or less. The lysate may be chemically processed to facilitate removal of detergents, proteins and lipids by the addition of salt. The precipitates formed by the addition of salt may be removed by centrifugation or filtration prior to transferring the sample to the matrix described above or may be transferred directly to the matrix without removing the precipitate. Preferred salts for the formation of precipitates are those which form insoluble complexes with both the detergents and proteins present in the sample. For example, potassium acetate is used in plasmid DNA preparations of the alkaline lysis type to form insoluble dodecylsulfate/protein complexes. Addition of transition metal ion salts in low concentration to the lysate in alkaline lysis purifications facilitates the formation of condensed precipitates, thus allowing the samples to be processed in a minimum volume. The concentration of transition metal ion salt is 0.01 - 0.1 M with a preferred concentration of 0.02 - 0.06 M. The DNA binding properties of the transition metal ions which are not apparent at low concentrations become apparent at concentrations above 0.1 M and significantly decrease DNA yield. The preferred transition metal ion salt is zinc chloride, although other salts of zinc and other transition metal ions may be useful in the method of the invention. The sample can be processed through the matrix by any method known in the art including but not limited to, vacuum

filtration, positive pressure filtration, gravity flow or centrifugation. The sample may be eluted from the matrix in a minimum volume using either water or a suitable low salt buffer. The buffer composition of the eluate may be changed by pre-equilibrating the matrix with the buffer which is to be used in subsequent processing of the sample. Typically the eluate contains a sample which is ready to use for further applications. If necessary, the DNA may be concentrated by precipitation from alcohol, ultrafiltration, or other methods known in the art.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1

A column was prepared in an 800 µl well, in a 96 well filter plate. Well dimensions were 1.087 inches long, tapered from a diameter of 0.274 inches at top to 0.204 inches at the base. The well was filled sequentially with 400 µl of an 80% suspension of Sephacryl® S-400 HR (Pharmacia) in water and 300 µl of an 80% suspension of Sephadex® G100 fine (Pharmacia) in water. Excess fluid was removed between additions by vacuum filtration and 200 µl of a 1:1 mixture of 80% Sephadex G100 and 3.3% poly(4-hydroxystyrene) beads (Advamax™ beads, Edge BioSystems) was added to the column. The column was then centrifuged at 1400 x g for 3 minutes to remove excess fluid volume. Lysed bacterial sample containing plasmid DNA was applied to the column and centrifuged at 1400 x g for 3 minutes to elute the sample. The plasmid was comprised of an unknown clone of human cDNA inserted in a pBLUESCRIPT vector (Stratagene). The host was a commercial strain of *E. coli* known as SOLR™ cells (Stratagene). Bacteria containing the plasmid were cultured overnight for 22 hours in 1 ml of terrific broth (Life Technologies) at 36° in the presence of 100 µg/ml ampicillin in a 96 well plate (2 ml plate with square well configuration) shaking at 325 rpm. The cell

suspension was pelleted by centrifugation for 5 minutes at 1400 xg. The bacteria were resuspended in 40 μl of water containing 300 μg/ml RNase A and 200 μg/ml lysozyme. After complete resuspension, the cells were incubated at room temperature for 5 minutes, lysed with 0.5 M NaOH containing 1.3% SDS, mixed by vortexing and allowed to sit for 8 minutes at room temperature. 20 μl of 2.5 M potassium acetate was added to the lysate, followed by 20 μl of a solution containing 1.45 M acetic acid, 0.04 M $ZnCl_2$ and 0.2 M TRIS base. The sample was mixed after each addition. The precipitated lysate was transferred to the column and centrifuged as described above. The eluate was free of detectable protein and detergent and contained approximately 25 μg of purified plasmid.

EXAMPLE 2

Porous beads used for binding proteins in the matrix of the invention in Example 1 were prepared as follows. Poly(4-hydroxystyrene)[MW 104,000] was dissolved in 50% aqueous ethanol at 8 mg/ml. The solution was pumped into flowing water at a rate of 80 ml/minute through an 18 gauge orifice mounted perpendicular to the flow of water in a closed system. Polymer was added until the concentration of alcohol reached 10-12%. The precipitate that formed was then concentrated by tangential flow filtration over a 30K membrane filter, and washed until the concentration of alcohol was less than 1%.

EXAMPLE 3

A column was prepared in a 760 μl microcentrifuge tube bucket 1.00 inches long, tapered from a diameter of 0.290 inches at top to 0.280 inches at the base. The bucket is supported at the base with an open plastic grid. Column components are retained by a porous polyethylene frit (15 - 30 micron nominal pore size) press fit into the bucket on top of the open plastic grid. The bucket was filled with 400 μl of an 80% suspension of Sephacryl® S-400 HR (Pharmacia) in water and 400 μl of an 80% suspension of Sephadex® G100 fine

(Pharmacia) in water. Excess fluid was removed between additions by vacuum filtration and 200 µl of diatomaceous earth suspension (BioRad, Quantum Prep Matrix) was added to the column. The column was then centrifuged at 1400 x g for 3 minutes to remove excess fluid volume. Lysed bacterial sample containing plasmid DNA was applied to the column and centrifuged at 1400 x g for 3 minutes to elute the sample. The plasmid was comprised of an unknown clone of human cDNA inserted in a pBLUESCRIPT vector (Stratagene). The host was a commercial strain of *E. coli* known as SOLR™ cells (Stratagene). Bacteria containing the plasmid were cultured, lysed and precipitated as in Example 1.. The precipitated lysate was transferred to the column and processed as described above. The eluate was free of detectable protein and detergent and contained approximately 26 µg of purified plasmid.

EXAMPLE 4

A column was prepared in a 760 µl microcentrifuge tube bucket 1.00 inches long, tapered from a diameter of 0.290 inches at top to 0.280 inches at the base. The bucket is supported at the base with an open plastic grid. Column components are retained by a porous polyethylene frit (15 - 30 micron nominal pore size) press fit into the bucket on top of the open plastic grid. The bucket was filled with 400 µl of an 80% suspension of Sephacryl® S-400 HR (Pharmacia) in water and 600 µl of an 80% suspension of Sephadex® G100 fine (Pharmacia) in water. Excess fluid was removed between additions by vacuum filtration and 15 µl of silica particles (Strataclean Resin, Stratagene, Inc) was added to the column. The column was then centrifuged at 1400 x g for 3 minutes to remove excess fluid volume. Lysed bacterial sample containing plasmid DNA was applied to the column and centrifuged at 1400 x g for 3 minutes to elute the sample. The plasmid was comprised of an unknown clone of human cDNA inserted in a pBLUESCRIPT vector (Stratagene). The host was a commercial strain of *E. coli* known as SOLR™ cells (Stratagene). Bacteria containing the plasmid were cultured, lysed and precipitated as

described in Example 1. The precipitated lysate was transferred to the column and processed as described above. The eluate was free of detectable protein and detergent and contained approximately 30 μg of purified plasmid.

EXAMPLE 5

A column was prepared in a 760 μl microcentrifuge tube bucket 1.00 inches long, tapered from a diameter of 0.290 inches at top to 0.280 inches at the base. The bucket is supported at the base with an open plastic grid. Column components are retained by a porous polyethylene frit (15 - 30 micron nominal pore size) press fit into the bucket on top of the open plastic grid. The bucket was filled with 400 μl of an 80% suspension of Sephacryl® S-400 HR (Pharmacia) in water and 600 μl of an 80% suspension of Sephadex® G100 fine (Pharmacia) in water. Excess fluid was removed between additions by vacuum filtration and 15 μl of silica particles (Strataclean Resin, Stratagene, Inc) was added to the column. The column was then centrifuged at 1400 x g for 3 minutes to remove excess fluid volume. Lysed bacterial sample containing plasmid DNA was applied to the column and centrifuged at 1400 x g for 3 minutes to elute the sample. The plasmid was comprised of an unknown clone of human cDNA inserted in a pBLUESCRIPT vector (Stratagene). The host was a commercial strain of *E. coli* known as SOLR™ cells (Stratagene). Bacteria containing the plasmid were cultured, lysed and precipitated as described in Example 1. The precipitated lysate was transferred to the column and processed as described above. The eluate was free of detectable protein and detergent and contained approximately 30 μg of purified plasmid.

EXAMPLE 6

A column was prepared in a 760 μl microcentrifuge tube bucket 1.00 inches long, tapered from a diameter of 0.290 inches at top to 0.280 inches at the base. The bucket is supported at the base with an open plastic grid. Column components are retained by a porous polyethylene frit (15 - 30 micron nominal pore size) press fit into the bucket on top of the open plastic grid. The bucket

was filled with 1500 μl of a 1:2 mix of an 80% suspension of crosslinked poly-4-hydroxystyrene beads prepared according to details provided in Example 7 and an 80% suspension of Sephadex® G100 fine (Pharmacia) in water. The gel was added in 2 steps to allow for limitiations of column volume. Excess fluid was removed between additions by vacuum filtration. The column was then centrifuged at 1400 x g for 3 minutes to remove excess fluid volume. Lysed bacterial sample containing plasmid DNA was applied to the column and centrifuged at 1400 x g for 3 minutes to elute the sample. The plasmid was comprised of an unknown clone of human cDNA inserted in a pBLUESCRIPT vector (Stratagene). The host was a commercial strain of *E. coli* known as SOLR™ cells (Stratagene). Bacteria containing the plasmid were cultured, lysed and precipitated as described in Example 1. The precipitated lysate was transferred to the column and processed as described above. The eluate was free of detectable protein and detergent and contained approximately 50 μg of purified plasmid.

EXAMPLE 7

Porous beads used for binding proteins in the matrix of the invention in Example 6 were prepared as follows. Poly(4-hydroxystyrene)[MW 104,000] was dissolved in 2 M NaOH at a concentration of 300 mg/ml. Butane-diol-diglycidyl ether (15 mole percent of PHS) was added and the solution incubated 20 hours at 55°C. The resulting gel was ground in a mortar and pestle and filtered through a series of sieves to give a bead with a 60 -120 particle distribution.

CLAIMS

1. A matrix for purifying a desired nucleic acid having a defined minimum molecular weight, said matrix having the ability to bind to proteins but not nucleic acids, said matrix retarding the migration through the matrix of components having a smaller hydrodynamic radius than the defined minimum molecular weight of the nucleic acid.

2. The matrix of claim 1 wherein the matrix is a homogeneous material consisting of porous particles comprised of a substance which binds proteins, wherein pores of the porous particle retard the migration of components having an effective hydrodynamic radius smaller than the defined minimum molecular weight.

3. The matrix of claim 1 wherein the matrix is a mixture of two or more materials.

4. The matrix of claim 3 wherein one of the materials binds proteins and one of the materials retards the migration of components having an effective hydrodynamic radius smaller than the defined minimum molecular weight.

5. The matrix of any of claims 1 to 4 wherein the matrix also binds detergent and or lipids.

6. The matrix of claim 3 wherein one of the materials binds proteins, one of the materials binds detergents, and one of the materials retards the migration of components having an effective hydrodynamic radius smaller than the nucleic acid with a defined minimum molecular weight.

7. The matrix of claims 5 or 6 comprising a hydroxylated aromatic polymer which binds proteins and detergents.

8. The matrix of claim 7 wherein the hydroxylated aromatic polymer is polyhydroxystyrene.

9. A method of purifying a desired nucleic acid having a defined minimum molecular weight, comprising the step of:

applying a preparation comprising the desired nucleic acids to a column containing a chromatography matrix having the ability to bind to proteins and to detergents but not to nucleic acids, said matrix retarding the migration through the matrix of components having an effective hydrodynamic radius smaller than the defined minimum molecular weight relative to the migration through the matrix of components having at least the defined minimum molecular weight.

10 The method of claim 9 wherein the preparation is a cell lysate.

11 The method of claim 9 wherein RNase has been added to the preparation.

12. The method of claim 9 wherein the preparation comprises less than 0.1 M salt.

13. The method of claim 9 wherein the matrix is a homogeneous material consisting of porous particles comprised of a substance which binds proteins and detergents, wherein pores of the porous particle retard the migration of components having an effective hydrodynamic radius smaller than the defined minimum molecular weight.

14. The method of claim 9 wherein the matrix is a mixture of two or more materials.

15. The method of claim 14 wherein one of the materials binds proteins and detergents and one of the materials retards the migration of components having an effective hydrodynamic radius smaller than the defined minimum molecular weight.

16. The method of claim 14 wherein one of the materials binds proteins, one of the materials binds detergents, and one of the materials retards the migration of components having an effective hydrodynamic radius smaller than the defined minimum molecular weight.

17. The method of claim 15 wherein the substance which binds proteins and detergents is a hydroxylated aromatic polymer.

18. The method of claim 17 wherein the hydroxylated aromatic polymer is polyhydroxystyrene.

19. The method of claim 10 wherein the cell lysate has been treated with a salt to form an insoluble complex with detergents and proteins.

20. The method of claim 19 wherein the salt is a transition metal ion salt.

21. The method of claim 20 wherein the concentration of the salt is 0.01 to 0.1 M.

22. The method of claim 20 wherein the concentration of the salt is 0.02 to 0.06 M.

23. The method of claim 19 wherein the insoluble complex is removed prior to applying to the column.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/21907

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/02, 21/04; B01L 11/00
US CL :422/101; 536/25.4, 25.41, 25.42
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 422/101; 536/25.4, 25.41, 25.42

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, CA, DERWENT
search terms: chromatography, DNA, protein-binding, hydroxylated aromatic, polyhydroxystyrene

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BOOS et al. On-line sample processing and analysis of diol compounds in biological fluids. J. Chromatog. 1988, Vol. 456, pages 93-104, especially 98 and 103.	1-23
Y	US 4,923,978 A (MCCORMICK) 08 May 1990, column 3, line 7 to column 5, line 27.	1-23
Y	EP 0 580 305 A2 (ADVANCED GENETIC TECHNOLOGIES CORPORATION) 26 January 1994, column 1, lines 17-52 and column 8, lines 52-57.	1-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer

Kenneth R. Horlick

Telephone No. (703) 308-0196